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Tubulin, a potential target for chemotherapy, plays an important role in different cellular functions including mitosis and cell division. Tubulin consists of mainly α and β subunits: each subunit exists in several isoforms. The distribution and expression of β-isoforms differ from tissue to tissue: β_{II} and β_{IV} are ubiquitously expressed while β_{III} expression is restricted to neurons and cancer cells. Moreover, $\alpha\beta_{III}$ has least affinity for anti-tubulin drugs. In this study, we correlated the expression and structural stability of β_{III}-tubulin in response to oxidative stress in cancer and drug resistant cells. We found that the cysteines in $\alpha\beta_{III}$ tubulin were less sensitive to oxidation compared to the cystines of $\alpha\beta_{II}$. We also found that β_{III}-tubulin is expressed in breast and other cancer cells but not in normal cell. Interestingly, the expression is highly elevated in drug resistant breast cancer cells and these cells experience higher oxidative stress than normal and other cancer cells. These data therefore, indicate that expression of β_{III}-tubulin in cancer and drug resistant cells is oxidative stress related. The structural stability, lesser susceptibility to oxidation in oxidative stress provides a strong rationale of over expression of β_{III}-tubulin in cancer and drug resistant cells.

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Introduction

Tubulin is a major and potential target for chemotherapy for breast and other cancers. Tubulin is a αβ heterodimeric protein and a major structural component of microtubules that play critical roles in mitosis: both α and β tubulin exists in several isoforms that are encoded by different genes. Among the isoforms, the β-tubulin isoforms are the most diverse in their sequences especially at their C-terminal regions. All these tubulin isoforms are expressed differently: β_{II} and β_{IV} are widespread in all our tissues while β_{III} has a restricted distribution (neuron) although it is elevated in many tumors. The ideal drug for therapy would be the one that would selectively bind to the heterodimer of β_{III}, $\alpha\beta_{III}$. Unfortunately, most successful anti-tumor drugs such as Taxol derivatives and *Vinca* alkaloids that are routinely used for the treatment of cancer bind least well to $\alpha\beta_{III}$ and best to either $\alpha\beta_{II}$ or $\alpha\beta_{IV}$. Not surprisingly, the breast cancer cells treated with these drugs increase the expression of β_{III} tubulin that causes drug resistance and creates a major obstacle in treating cancers. Our recent data indicate that the breast and other cancer cells with elevated β_{III} tubulin have elevated levels of reactive oxygen species (ROS) compared to normal cell. We also found that cysteines in bovine brain $\alpha\beta_{III}$ tubulin are less susceptible to alkylation than the most abundant isoform $\alpha\beta_{II}$ indicating that $\alpha\beta_{III}$ is less susceptible to oxidize. Therfore, the major objective in this proposal to establish a direct relationship between β_{III} tubulin expression and the redox state of cancer cells. We hypothesize that cancer cells increase expression of β_{III} tubulin to protect microtubules from the oxidative stress caused by reactive oxygen species. The over expression of β_{III} that has lower affinity for anti-tumor drugs and less reactivity to reactive oxygen species results in the development of drug resistance. The result of this work will help in understanding the importance and role of the β_{III} isoform and the reactive oxygen species in the progression of drug resistance in breast cancer cells.

Body:
The central hypothesis of this proposal is to test that the development of drug resistance by breast cancer cells is accompanied by increased expression of the β_{III} isoform and increased oxidizing conditions in the cells.

The statement of the work for the first year (6/1/02 to 5/30/03) is described below:

A. We will purify the β-tubulin isoform namely the most abundant one $\alpha\beta_{II}$ and the $\alpha\beta_{III}$ from the purified unfractionated bovine brain αβ tubulin by affinity column chromatography and will measure the surface cysteine residues of each dimer at their native states.

Microtubules were purified from bovine cerebra by a cycle of assembly and disassembly and the tubulin was purified thereform by phosphocellulose chromatography following the procedure of Fellous et al (1). Experiments were performed in the following buffer: 50 mM Pipes buffer pH 6.9, 0.5 mM GTP, 0.5 mM $MgCl_2$ and 1 mM EGTA. The individual dimer of the β-tubulin isoform ($\alpha\beta_{II}$ and $\alpha\beta_{III}$) was purified from the unfractionated αβ tubulin by affinity column chromatography as described (2,3) and the protein concentration was measured by method of Lowry et al (4). In brief, for purification of $\alpha\beta_{II}$-tubulin, phosphocellulose purified tubulin was loaded on the anti-β_{III} column and collected the unbound fractions containing a mixture of $\alpha\beta_{II}$ anf $\alpha\beta_{IV}$ tubulin. The unbound fraction was then loaded on an anti-β_{IV} column to collect the unbound fraction that was pure $\alpha\beta_{II}$ tubulin. To purify the $\alpha\beta_{III}$ tubulin, the unfractionated tubulin was loaded on the anti-$\alpha\beta_{II}$ column and collected the unbound fraction that contained $\alpha\beta_{III}$ and $\alpha\beta_{IV}$. The unbound fraction was then loaded to anti-$\alpha\beta_{IV}$ column and collected the unbound fraction that was pure $\alpha\beta_{III}$ tubulin. We have developed **a surface cysteine scanning assay** in measuring the differential exposure of the cysteine residues in different β-tubulin isoforms ($\alpha\beta_{II}$ and $\alpha\beta_{III}$) in their native states **(Figure 1)**. In brief, we treated the aliquots (500 µl) of β-isoform purified tubulin dimers ($\alpha\beta_{II}$ and $\alpha\beta_{III}$) (5 µM) with different concentrations of urea (0, 0.2, 0.4, 0.6, 0.8, 1, 3M) followed by incubation with 500 µM *N*-ethylmaleimide (cysteine modifying agent) in the dark at 37 °C for 45 min to modify the surface and accessible cysteine residues in tubulin. After the reaction was over, samples were treated with 500 µM dithiothreitol (DTT), a thiol containing reducing agent, to quench the excess *N*-ethylmaleimide in the reaction mixture. The tubulin samples were then precipitated with equal volume of cold 20% TCA (v/v) followed by washing at least four times with chilled ethanol and ethylacetate (1:1) to remove the free *N*-ethylmaleimide, if present. Then precipitated tubulin proteins were dissolved in Pipes buffer containing 6M urea and incubated with (500 µM) [^{14}C]N-ethylmaleimide at 37 °C for 45 min in the dark to modify the cysteine residues that were not exposed onto the surface in the native state of tubulin. The protein concentration was measured followed by running equal amount of protein (10 µg) in SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). From the Top panel of Figure 1, we found that cysteines in $\alpha\beta_{II}$ tubulin were mostly exposed on the surface with and without perturbation of the structure (lanes 1-4) as we detected very little incorporation of [^{14}C]*N*-

ethylmaleimide into protein. As the comparison between untreated and treated tubulin samples with urea showed no difference in incorporation in $\alpha\beta_{II}$ tubulin suggesting strongly that cysteines were in fact surface-accessible. In case of $\alpha\beta_{III}$ tubulin, a significant proportion of the cysteine residues were unavailable on the surface as marked increase in incorporation of [^{14}C]*N*-ethylmaleimide occurred in absence and presence of urea (bottom Panel, lanes 1-7). Even in the presence of 3M urea (lane 7), a little incorporation was detected in the α-subunit. Therefore, these data clearly suggest that $\alpha\beta_{III}$ tubulin is structurally stable and the cysteines in $\alpha\beta_{III}$ are not readily available for oxidation compared to $\alpha\beta_{II}$ tubulin in the native and partially unfolded state.

B. We will grow normal and different established breast cancer cell lines to measure the level of expression of β-tubulin and its different β-tubulin isoforms namely β_{II} and β_{III}. We will also measure the level of reactive oxygen species in normal and cancer cell lines.

The monoclonal antibodies JDR.3B8 and SDL.3D10 specific, respectively, for the β_{II} and β_{III} isoform of tubulin were prepared as previously described (2,3). The peptides used as immunogens for raising antibodies were CEGEEDEA and CESESQGPK, respectively for β_{II} and β_{III} tubulin. Immunoblotting of gels was carried out as previously described (3) except that the secondary antibody, instead of being radioactive, was conjugated with horseradish peroxidase. In brief, normal (A-10), breast (MCF-7, MDA435 and BT5492) and other cancer cells (PC-3, DU145, HeLa, Hs578T) and a transformed cell (HUVEC) grown on Petri dishes were lysed with ice cold buffer, containing 20 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate, 2 mM EGTA and complete proteinase inhibitor cocktail tablets and sonicated by 30 sec cycles. Protein concentration was determined by Lowry before polyacrylamide gel electrophoresis (PAGE) carried out in 7.5% SDS-discontinuous gels. 30 μg of protein extract was loaded in each lane of the gel. Proteins were electrotransferred to nitrocellulose membranes, which were stained with Ponceau S to verify the amount of protein transferred in each lane. The nitrocellulose paper was blocked with 5% skim in PBS and incubated in a 1:10,000 dilution of the β_{II} and 1:5000 dilution of the β_{III} antibody, overnight at 4 °C. The blots were washed at least three times (20 min each) in PBS and incubated for 1 hr in a 1:5000 dilution of peroxidase-conjugated goat antimouse IgG. Positive bands were visualized with the Amersham enhanced chemiluminiscence ECL kit. In **Figure 2**, we measured the expression of β, β_{II} and β_{III} tubulin isoforms in nontransformed and cancer cells. The top panel showed the expression of β-tubulin in different cell lines. (+) was the αβ tubulin that was used for positive control and rests were the extracts from different cell lines (A10, BT549L, DU145, HeLa, Hs578t, HUVEC, MCF-7, MDA-MB-435 and PC-3). The middle panel showed the expression of βII in different cell lines. As $\alpha\beta_{II}$ constitutes 52% in unfractionated αβ tubulin and is a major isoform expressed ubiquitously in all tissues, we detected significant amount of β_{II} in all cancer as well as in A-10 cells (smooth muscle) that is considered as normal cells. Interestingly, we did not detect the β_{III} tubulin in A-10 but detected in all breast and other cancer cell lines supporting the earlier observations (5-8) that the expression of β_{III} tubulin is restricted to transformed and cancer cells. Moreover, we detected 29 times more β_{III} in BT5492 compared to MDA435.

We measured the level of reactive oxygen species (ROS) in breast and other cancer cell lines namely, A-10, MCF-7, HeLa, BT549L and PC3 **(Figure 3).** DCFA (dichlorofluorescein diacetate) was used as a probe in measuring the level of intracellular ROS. The excitation and emission wave length for DCFA was 504 and 524 nm, respectively. In brief, after cells grew in confluent, cells were treated with 20 μM DCFA for 30 min in the dark at room temperature followed by washing cells in Petri dishes with PBS at least four times. Then cells were harvested, sonicated by 30 sec cycles in presence of Pipes buffer containing 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton-X and protease inhibitors and centrifuged at 12000g at 4 °C for 30 min. The protein concentration in the supernatant of each of the sample was measured. Equal amount of protein was taken (50 μg) and the fluorescence was measured at 524 nm.

We also measured the oxidized status in normal, breast cancer and drug resistant cell lines to support the hypothesis that breast cancer and drug resistant cell lines have higher level of reactive oxygen species. I compared two breast cancer cell lines: MDA435 and BT5492 with normal smooth muscle cells (A-10). The BT5492 cells over express 29 times as much as β_{III} as do the MDA435 cells while A-10 has no detectable β_{III} as I mentioned earlier (Figure 2). When DCFA was used to measure ROS in these cells, we found a high level of ROS in BT5492 compared to MDA435. Moreover, MDA435 has much more ROS than do the A-10 cells **(Figure 4).** Interestingly, BT5492 cells, unlike MDA435, are highly resistant to vinblastine, taxol and cryptophycin.

C. We will grow breast cancer cells in the presence of artificial ROS generators to measure the level of expression of expression of different β-tubulin isoforms. We will also measure the level of reactive oxygen species.

We exposed cells to different doses of ionizing radiation to see the effect of ROS on the expression of β-tubulin isoforms as I proposed that oxidative stress would elevate the level of expression of β_{III} tubulin. This part is in progress. However, we measured the effect of ionizing radiation on the ROS production in breast cancer cell (MCF-7). The rationale in selecting MCF-7 cells in measuring radiation-induced ROS production was we detected maximum intracellular ROS production in MCF-7 among all other cell lines that suggest that MCF-7 had higher endogeneous oxidized environment. We found that the fluorescence of cell extracts at 524 nm was increased with escalating doses of radiation when cells were pre-incubated with DCFA for 30 min followed by radiating cells with different doses and harvested cells after 24 hrs. We also noticed a significant increase in fluorescence in the extracts of cells that were first radiated with different doses, then growing cells for 24 hrs followed by exposing to DCFA for 30 min before harvesting **(Figure 5)** although dose-dependent increment of fluorescence was not observed. This experimental result suggests that the ionizing radiation generated a significant level of ROS production that was even detected after 24 hrs. This artificial oxidative stress is likely to elevate the expression of β_{III} tubulin.

Key Research Accomplishments

The following observations were made from this research

1. $\alpha\beta_{III}$ tubulin is structurally more resistant to unfold than $\alpha\beta_{II}$ tubulin and the cysteines in $\alpha\beta_{III}$ tubulin are less available to oxidation compared to the cysteines of $\alpha\beta_{II}$ tubulin as significant differential incorporation of [^{14}C]iodoacetamide was detected between the two isoforms in native and partially unfolded state.

2. The level of expression of β_{III} was measured in normal smooth muscle (A-10) and in breast and other cancer cell lines to support the existing concept that β_{III} is elevated in cancer and highly elevated in drug resistant cancer cells. We found that β_{III} was not expressed in normal cell but elevated in all transformed and cancer cells, especially in breast cancer cells namely MCF-7, MDA435 and BT5492 cells. Interestingly, the level of expression was elevated maximum in BT5492 that is highly resistant to tubulin-targeted drugs such as Taxol, *Vinblastine*. This finding suggests that the expression of β_{III} and drug resistance in cancer cell is directly correlated.

3. We also found in general that cancer cells experience more oxidative stress compared to the normal cell. Moreover, the drug resistant cells experience maximum oxidative stress compared to other cells.

4. As cancer and drug resistant cells experience higher oxidative stress, they need structurally resistant tubulin dimer for their survival, growth and maintaining of cellular functions. The rationale for selection of $\alpha\beta_{III}$ tubulin by cancer and drug resistant cells was that the cysteines in $\alpha\beta_{III}$ tubulin are least sensitive to oxidation and $\alpha\beta_{III}$ tubulin is structurally stable than other isoforms. In case of normal cell, the oxidative stress is markedly low compared to the cancer cells, so they do not require β_{III} tubulin for their function. That's why; normal cells do not express β_{III} tubulin.

Reportable Outcomes:

These data provide important information on the regulation of expression of β_{III} tubulin in response to oxidative stress in cancer and drug resistant cells. I am writing manuscript on this observation and preparing abstract for cancer meeting.

Conclusion:

These data all together strongly propose a probable mechanism of expression of β_{III} tubulin in breast and other cancer and drug resistant cells. I used different cancer cell lines to establish the fact that the observation that I reported was not restricted to breast cancer cells but rather was a general phenomenon in different cancer cells. From first year of our research, we found that breast cancer, other cancer (prostate) and drug resistant cells experience higher endogenous oxidative stress compared to the stress observed in normal smooth muscle cell (A-10). I initially proposed to use MCF-10F as normal cells. I had a hard time to have enough cells to do *in vitro* experiments. Therefore, I used A-10 to simply show that proteins in normal cells generally experience low oxidative stress compared to proteins of cancer cells and the expression of β_{III} was strictly restricted to cancer cells.

In response to high oxidative stress in cancer and drug resistant cells, β_{III} tubulin was expressed in cancer and drug resistant cells. Now question comes why cancer cells chose β_{III} tubulin in response to oxidative stress? Using surface cysteine scanning assay and the data obtained from others (9) suggested that $\alpha\beta_{III}$ tubulin was structurally resistant in denaturing environment as determined by exposure of the cysteine residues. Since cystines were not available on surface due to structural integrity of $\alpha\beta_{III}$ tubulin, they were not easily alkylated or oxidized by the iodoacetamide. This observation promptly suggested that the cysteines of $\alpha\beta_{III}$ tubulin would not be readily oxidized in cells even experiencing high oxidative stress. However, the cysteines of $\alpha\beta_{II}$ were more sensitive and available on surface. This unique property of $\alpha\beta_{III}$ tubulin probably assisted cancer cells to build stable microtubules to survive and maintaining cellular functions in adverse situations such as in oxidative stress.

As I found the expression of β_{III} tubulin was highly elevated in drug resistant cell such as BT5492 compared to other breast cancer cells and was resistant to Taxol and *Vinca* alkaloids, I am planning to isolate $\alpha\beta_{III}$ tubulin from BT5492 by affinity chromatography to measure the affinity for anti-tubulin drugs *in vitro* and also to compare the affinity for drugs with β_{III}-depleted tubulin. As $\alpha\beta_{III}$ has least affinity for different tubulin-directed drugs (10-14), it is predicted that $\alpha\beta_{III}$ -depleted tubulin fraction that will contain mainly $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ will have higher affinity compared to the $\alpha\beta_{III}$ tubulin. These data will definitely establish the role of $\alpha\beta_{III}$ tubulin in anti-tublin drug resistance in BT5492 cells.

I am also planning to establish that $\alpha\beta_{III}$ tubulin isolated from radiated BT5492 cells have least sensitive to oxidative damage compared to the level of damage in $\alpha\beta_{II}$ tubulin isolated from same cell line. To measure the damage, I will measure the carbonyl residue formation in tubulin. As ionizing radiation generates ·OH (hydroxyl) radical, there is high chance of formation of carbonyl residues in tubulin as it is established

phenomenon that ·OH radical causes carbonyl formation by metal catalyzed reaction (15-16). I will also measure the level of expression of β_{III} and other isoform, namely β_{II}. The prediction is that $\alpha\beta_{III}$ tubulin will be least oxidized by carbonyl residues compared to the $\alpha\beta_{II}$ tubulin and the level of expression of β_{III} might increase with increasing doses of radiation. These results will definitely help in drawing conclusion that β_{III} tubulin plays major role in cancer and drug resistant cells.

"So what section": The data obtained from this research will provide strong evidence in understanding the function of $\alpha\beta_{III}$ tubulin in cancer and drug resistant cells. This information along with the crystal structure data will help in designing drugs that will bind tightly to $\alpha\beta_{III}$ tubulin and least to other isoforms that express ubiquitously. The positive point in designing drug against $\alpha\beta_{III}$ tubulin is that the drugs will have least cytotoxic effect to normal cells as the expression of β_{III} is restricted to cancer and drug resistant cells.

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Curriculum Vitae

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Education:

Ph.D. in Plant Biochemistry, Bose Institute, University of Calcutta, Calcutta, India, 1993.

Title: Physicochemical characterization of tubulin and its molecular organization in Mimosa pudica.

M.Sc. in Biochemistry, University of Calcutta, Calcutta, India, 1986.

B.Sc. in Chemistry, University of Calcutta, Calcutta, India, 1984.

Grants and Awards:

Recipient of Barbara H. Bowman Post Doctoral Scholarship Award for 1997.

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Principal investigator: Dr. Asish Ray Chaudhuri
Total cost: $194,.437 (approximately)
Period: Junel, 2002 - May 31, 2004.

Professional Affiliation:

Member of The American Society of Cell Biology (1994 to present)
Member of San Antonio Cancer Institute (February 2000 to present)
Member of Aging Research and Education Center, San Antonio
(February 2000 to present)
Scientific Advisory Board Assistant of SAGE KE (*Science* online issue of Aging Knowledge Environment) (February 2002 to present)

Invited Lectures:

February, 1999	Bionumerik Pharmaceutical Inc., San Antonio.
December, 2000	Department of Medicine, UTHSCSA.
January, 2001	Wyeth & Ayest Pharmaceuticals, New York.
January 2001	Department of Radiation Oncology, UTHSCSA.
February 2001	Aging Research and Education Center, UTHSCSA.
February 8, 2002	Clinical Radiation Oncology Lecture Series, UTHSCSA.
February 13, 2002	Mini symposium organized by Department of Radiation Oncology, UTHSCSA.

Work Experience:

Research Instructor
Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas (February 2000 to present)

Senior Research Associate
Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas (November 1996 to January 2000)

Post Doctoral Fellow
Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas (March 1994 to November 1996)

Research Associate
Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi (May 1993 to February 1994)

Period (March 1994 to present)

Studied the role of ROS (reactive oxygen species) and the different antioxidants on drug binding and ß-tubulin isoform expression in normal and cancer cells.

Studied the effect of ROS formed by low doses of ionization radiation on conformation and drug binding to tubulin in MCF-7 breast cancer cells.
Developed ***a novel disulfide detection method*** to detect the intrinsic disulfide bonds formed in tubulin and other proteins at different oxidized state of cells.

Studied the mechanism and site of interaction of the tumor suppressor protein Fhit (Fragile Histidine triad) with tubulin-microtubule system.

Developed ***fluorometric methods*** to study the quantitative interaction of different antitumor drugs and proteins with tubulin.

Developed ***a novel footprinting method*** to determine the binding sites as well as the domain affected by drugs on tubulin.

Developed ***a novel surface cysteine scanning method*** to map selectively the surface as well as the internal cysteine residues of functional proteins at reducing and oxidized state.

Developed ***a novel surface hydrophobic site detection method*** in determining proteins that are structurally altered/unfolded in aging and in pathological situations.

Period (May 1993 to February 1994)

Determined the nature of glycosylation in silk proteins of larvae.

Studied the functional properties of glycosylated molecules attached to the silk protein.

Ph. D. (January 1987 to December 1992)

Studied the biochemistry, genomic organization and the physical aspects of tubulin of *Mimosa pudica* plant.

Research Interest:

Detection and characterization of proteins that are susceptible to oxidation in aging and in tumorogenesis using a novel proteomic approach.

Tissue-specific oxidation of proteins in normal and aging cells.

Modulation of oxidation in proteins by antioxidants in cancer and aging cells.

Oxidative stress induced modulation of GTP binding on G-proteins in aging and cancer cells.

Effect of low doses of ionization radiation and in combination with antioxidants on the expression and the conformation of ß-tubulin of normal and multi-drug resistant cells.

Search and synthesize for new molecules that would protect proteins in cells from free radical damage in aging and radiation therapy.

Reviewer: Biochemical Pharmacology

Publications:

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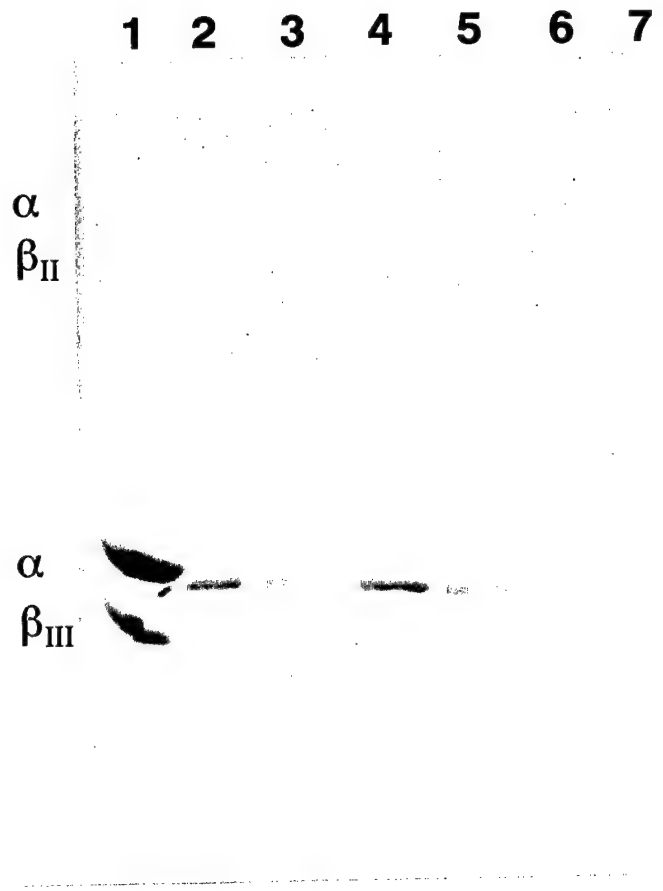

Figure 1: Exposure of the Surface Cysteines in $\alpha\beta_{II}$ and $\alpha\beta_{III}$ Tubulin in Different Urea Concentrations

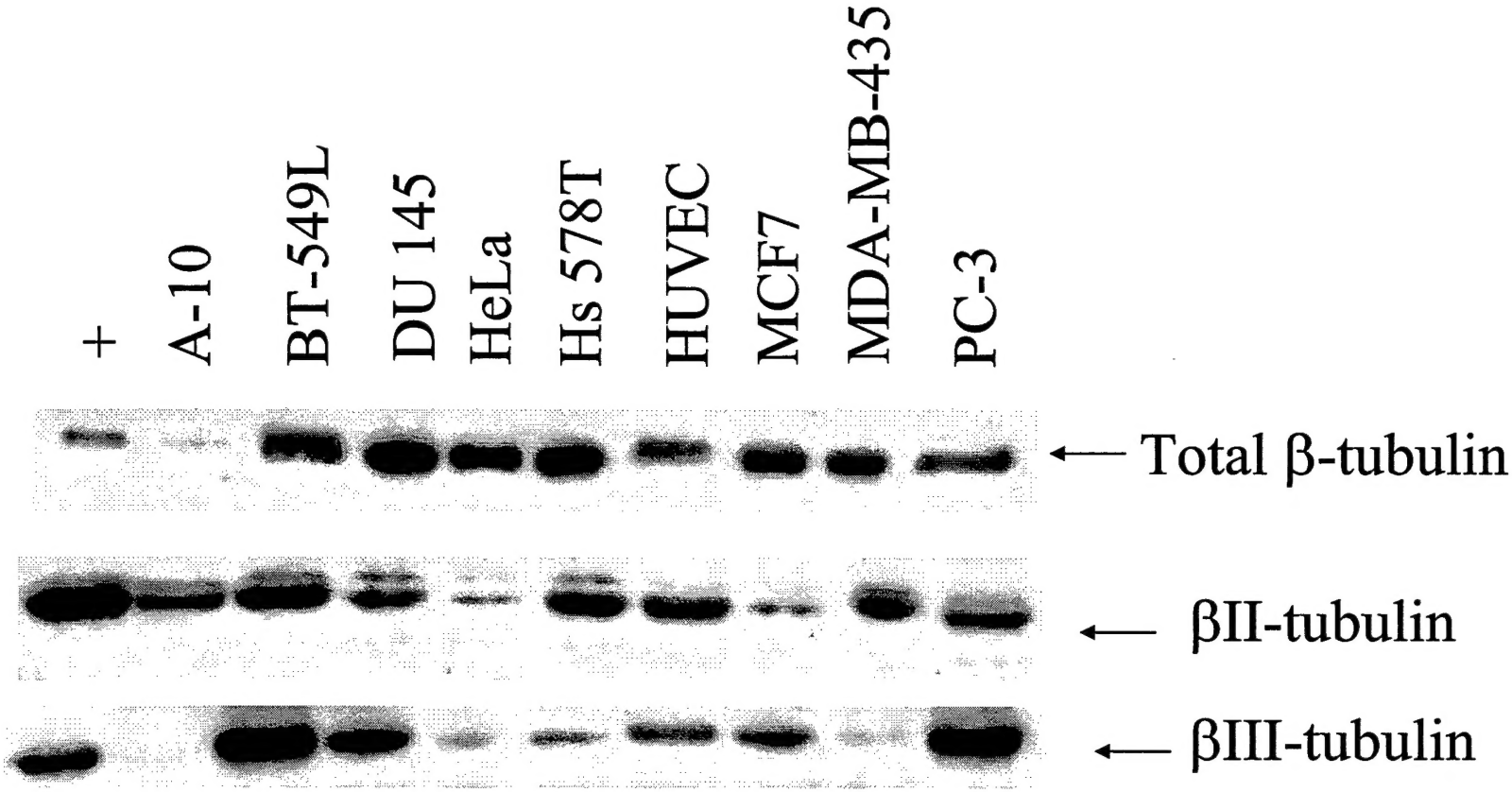

Figure 2: Western Blot Analysis of the Expression of β-Tubulin Isoforms in Nontransformed and Cancer Cell Lines

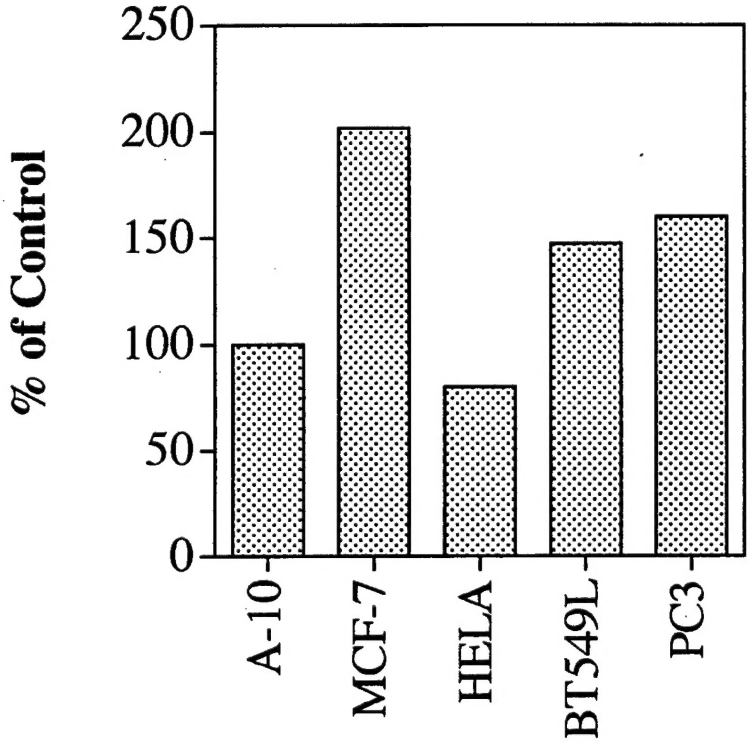

Figure 3: Measurement of Oxidized Status by DCFA in Normal and Cancer Cell Lines

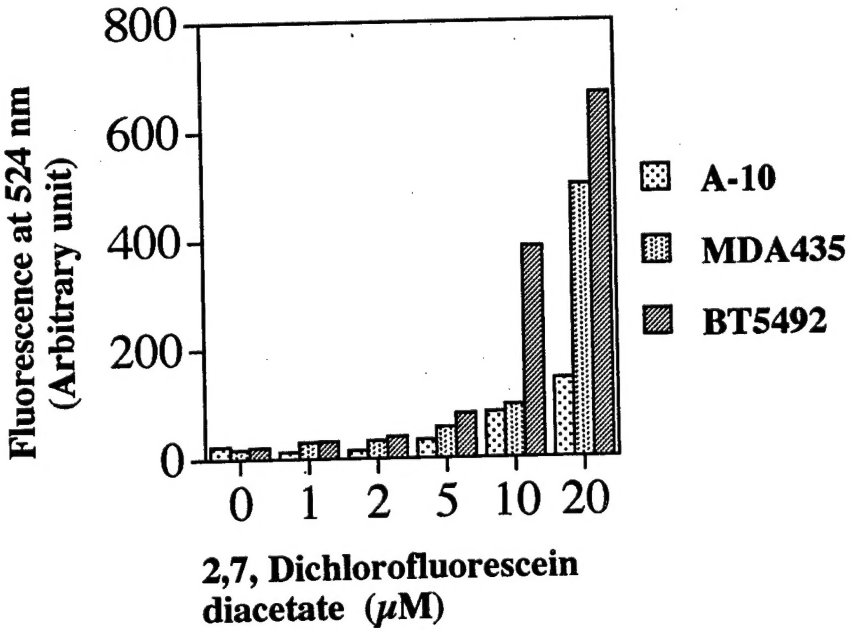

Figure 4: Measurement of Oxidized Status by DCFA in Normal , Breast Cancer and Drug Resistant Breast Cancer Cell Lines

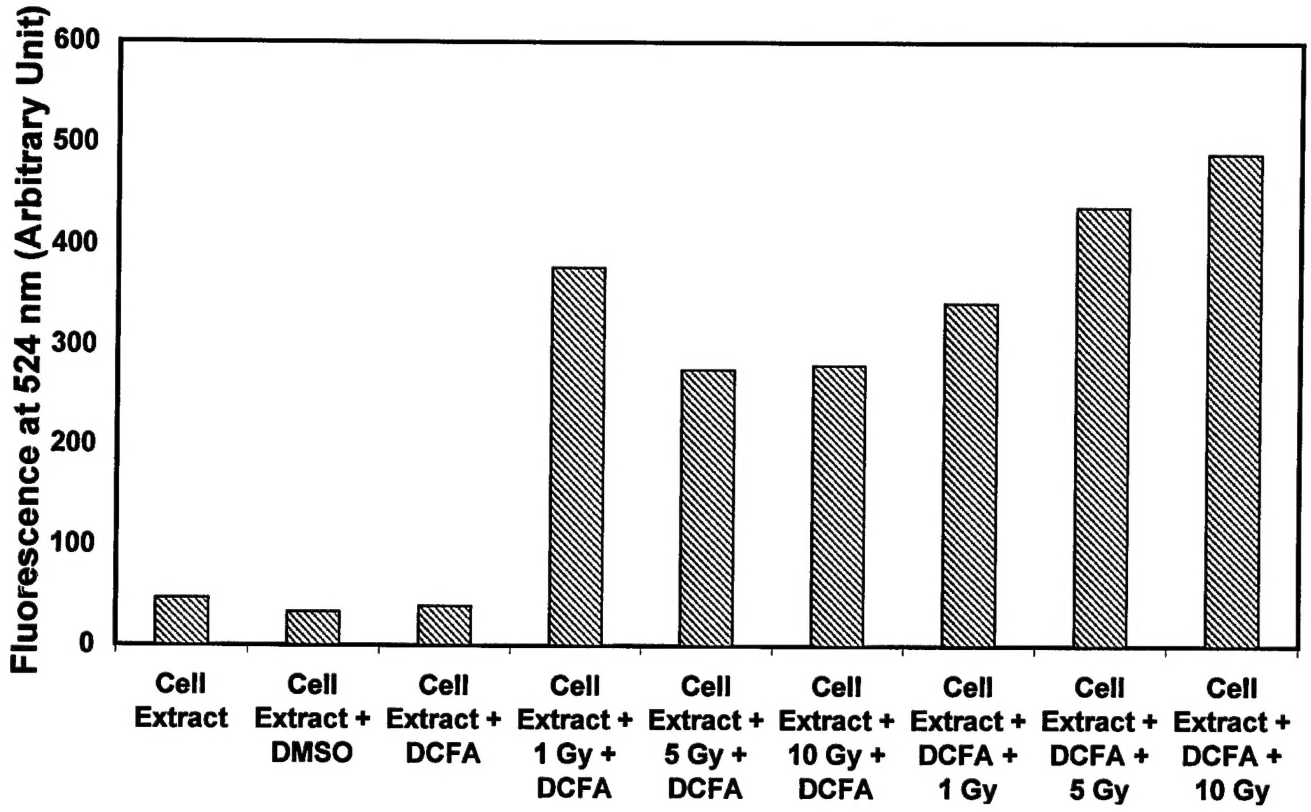

Figure 5: Effect of Different Doses of Ionizing Radiation On Production of ROS in MCF-7 Cell Line